High performance liquid chromatographic separation of anabolic oestrogens and ultraviolet detection of 17β-oestradiol, zeranol, diethylstilboestrol or zearalenone in avian muscle tissue extracts

A multi-residue analysis was developed to discern oestrogens (17β-oestradiol or zeranol) used as anabolic drugs and diethylstilboestrol (DES) from the mycotoxins zearalenone and zearalenol. A mixture of 17β-oestradiol, oestrone, DES, zeranol, zearalenol, zearalenone and zearalanone was analysed using a 5 μm silica column with gradient elution from hexane to hexane : methanol : 2-propanol, 40 : 45 : 15 (v/v) and ultraviolet detection at 280 nm. Only six peaks were obtained since zearalenone and zearalanone had identical elution times. Minimum detectabilities were 5–10 ng for the standards. Chicken muscle tissues (2·5 g) were extracted with acetone : water (95 : 5, v/v). The extracts were spiked with oestradiol and zeranol at 50, 100 or 200 ppb; and DES or zearalenone at 50 ppb; cleaned-up through dual columns of basic alumina and phosphate exchanged AGMP-1 resin and analysed by high-performance liquid chromatography with ultraviolet detection (HPLC–UV). Minimum detectability was 10 ng for oestradiol, zeranol, DES or zearalenone when tissues were spiked at 50 ppb. Recoveries were ⩾86%, ⩾83%, 60% and 100% for oestradiol, zeranol, DES and zearalenone, respectively.

Introduction

There is a need to develop a practical and reliable method to monitor the residues of anabolic drugs in tissues and fluids of food-producing animals. Anabolic oestrogens, 17β-oestradiol, 17β-oestradiol benzoate and zeranol are used as growth promoting agents and to increase feed efficiency in cattle and sheep, while 17β-oestradiol monopalmitate enhances uniform fat distribution in chicken roasters (*Code of Federal Regulation* 1984, Herrick 1984). Diethylstilboestrol (DES) is banned in USA, Canada and most EEC (European Economic Community) member countries but has been found to be used illegally in veal calves. 17β-Oestradiol, a steroid hormone naturally present in man and animals, may be a possible health hazard to consumers if ingested in quantities greater than physiological levels (IARC 1979). No zeranol residue is allowed in edible tissues of treated animals (*Code of Federal Regulation* 1984) but the presence of naturally occurring mycotoxins (zearalenone and its metabolite zearalenol) can interfere with the analysis. Zeranol is industrially produced from zearalenone (Baldwin 1983). Zeranol and its metabolite, zearalanone, have similar structure to those of the mycotoxins. These mycotoxins can be elicited by *Fusarium* moulds in feeds and subsequently ingested by farm animals. The presence of zearalenone and zearalenol in foods and feeds and their oestrogenic effects on farm animals were reported by Mirocha *et al.* (1974, 1977, 1979), Hurd (1977) and Betina (1984). Hagler *et al.* (1980) mixed zearalenone with ruminant feeds and found zearalenone and β-zearalenol transmitted in milk. Scott *et al.* (1978) also reported the presence of zearalenone in corn-based cereal

foods. Availability of a reliable and sensitive method to monitor these contaminants will help ensure the delivery of safe and wholesome meat and poultry products to consumers.

High-performance liquid chromatography (HPLC) separation with ultraviolet or fluorescence detection was developed mostly for analysis of single compounds or multi-component systems for steroids, oestrogens, mycotoxins or anabolic drugs. The use of HPLC for analysis of steroids was reviewed by Heftman and Hunter (1979). Ryan (1976) reviewed its use in the analysis of hormone residues including DES, oestradiol and zeranol. Recently, Roos (1980), Loesch and Simon (1983) and Lin (1984) reported HPLC separation and identification of oestrogens by normal, reversed or partition systems. Other investigators also reported determination of zearalenone and α-zearalenol in porcine plasma (Turner *et al.* 1983), in chicken fat, heart and kidney (Trenholm *et al.* 1981) and in animal chow (Holder *et al.* 1977). Hagler *et al.* (1980) determined zearalenone and β-zearalenol by reversed phase HPLC–UV in bovine and ewe milk, and Palysiuk *et al.* (1980) measured zearalenone and zearalenol in sow's milk. Frishkorn *et al.* (1978) reported a simultaneous detection of zeranol and its metabolite zearalanone in spiked meat tissues, while Grohman *et al.* (1982) developed a multi-residue analysis of anabolics (oestradiol, DES, zeranol, dienoestrol, hexoestrol and ethinyloestradiol) in meat. Jansen *et al.* (1984) also developed separation techniques for anabolics, stilbene derivatives, zeranol and derivatives in bovine urine for subsequent analysis by radioimmunoassay, thin layer chromatography or gas chromatography–mass spectrometry. Reported detectability by these methods were from low ng to μg when the oestrogens were present in parts per billion to parts per thousand. There is no reported HPLC–UV multi-residue method to analyse the anabolic drugs, their metabolites and the mycotoxins simultaneously. Therefore, there is a need to develop a multi-residue method to discern these classes of oestrogenic compounds.

This study presents (*a*) the development of a normal phase HPLC method to separate oestradiol, oestrone, zeranol, DES, zearalenone, zearalanone and zearalenol with ultraviolet detection at 280 nm; and (*b*) the use of our recently developed dual columns of basic alumina and phosphate exchanged AgMP-1 resin to clean up acetone extracts of avian muscle tissue.

Methods and materials

Reagents and equipment

We used basic alumina (80–200 mesh, Brockman activity 1), sea sand, precision glass beads (3 mm), diethyl ether, anaesthesia grade (100 ml), and an IEC Centra-R7 centrifuge, AgMP-1 anion exchange resin (100–200 mesh) and polypropylene Econo-Column from Bio-Rad Laboratories, Richmond, California; acetone, toluene, hexane, methanol and 2-propanol (distilled in glass) from Burdick and Jackson, Muskegon, Michigan; polypropylene pipette tips (5 ml) from Rainin Instrument Company, Woburn, Massachusetts; 17β-oestradiol and oestrone from Sigma Chemical Co., St. Louis, Missouri; DES and acetic acid (Gold Label) from Aldrich Chemicals Co., Milwaukee, Wisconsin; zeranol, zearalanone, zearalenone and α-zearalenol from International Minerals and Chemicals Corp., Terre Haute, Indiana; a Polytron equipped with a PTA 10 generator from Brinkman Instrument, Westbury, New York; a Sonicator with microtip from Heat Systems, Plainview, New York; and a IKA rotary shaker from Tekmar Co., Cincinatti, Ohio.

HPLC apparatus. We used a silica column (5 μm particles, 25 cm × 4·6 mm i.d.) from Supelco; injector (Model 210) from Altex, Berkeley, California; gradient programmer (Model 334), solvent delivery system (Model 420) and pumps (Model 110A) from Beckman, Fullerton, California; UV–VIS variable wavelength detector (Spectroflow 773) from Kratos Analytical Instruments, Ramsey, New Jersey; and Recordall Series 5000 from Fisher Scientific Co, King of Prussia, Pennsylvania.

HPLC separation of standards

The elution patterns of 17β-oestradiol, oestrone, DES, zeranol (zearalanol), zearalenol, zearalenone and zearalanone (figure 1) were determined by HPLC using a silica column. The conditions to maximize the separation of the seven compounds were determined with gradient elution of mobile phases A (hexane) and B (hexane : methanol : 2-propanol). Mobile phase B ranged in proportion from 80 : 15 : 5 to 40 : 45 : 15 (v/v). The elution patterns of oestradiol and oestrone were used as reference standards.

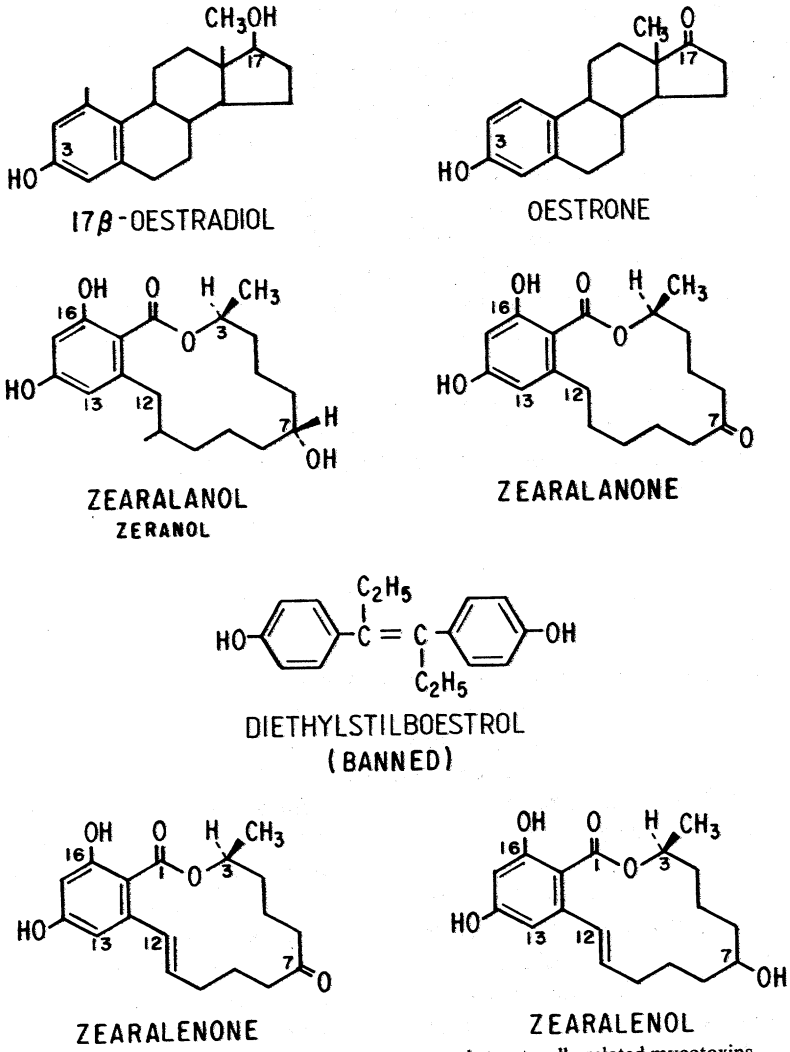

Figure 1. Structure of anabolic oestrogens and structurally related mycotoxins.

Sample preparation and clean-up

Chicken muscle tissues (2·5 g) were homogenized in 10 ml acetone using the Polytron for 20 s followed by sonication for 5 min and centrifuged at 3200 rpm, and the supernate was decanted into silanized conical tubes. Another 8 ml acetone were added to the pellet and the extraction process was repeated and the supernates of each samples were pooled. The supernates of three sets of 2·5 g tissue samples were spiked with a binary mixture of oestradiol and zeranol containing 125, 250 or 500 ng of each compound. Another set of samples was spiked with 125 ng each of DES or zearalenone. An unspiked extract was used as a control. The spiked extracts were transferred into a 5 ml polypropylene pipette tip containing approximately 1·5 g of alumina arranged in tandem with the Econo-Column filled with phosphate-exchanged AGMP-1 resin up to the 2·0 ml mark or an equivalent of 1·0 g dry resin (Medina and Schwartz 1986). The extracts were allowed to percolate by gravity through both columns. The columns were then washed with 4 × 1 ml acetone:water (95:5, v/v). The alumina column was removed and the wall of the ion-exchange column was rinsed with 1 ml acetone:water (95:5) and the oestrogens were eluted with 4 × 1 ml 10% acetic acid in acetone and collected in silanized 15 ml conical tubes. The acetic acid/acetone effluents were blown down to dryness with nitrogen at 40°C. Distilled water (0·5 ml) was added and the aqueous mixture was extracted twice with 2 ml ether. The ether extracts were blown down to dryness with nitrogen.

HPLC analysis of tissue samples

The dried ether extract was reconstituted with 250 μl of mobile phase B as the oestrogens were not completely soluble in hexane at ambient temperature. Aliquots of 20 μl were analysed by HPLC using the optimum conditions selected to separate the seven oestrogenic compounds.

Results and discussion

The normal phase HPLC separated the seven estrogens into six peaks. Zearalenone and zearalanone were eluted as single peak (figure 2). The optimum conditions were gradient elution (15 min) from 100% mobile phase A (hexane) to 40% mobile phase B (hexane:methanol:2-propanol, 40:45:15, v/v) at a flow rate of 2·0 ml/min and the peaks were detected at 280 nm. All seven compounds were eluted in less than 15 min. However, because of the increased amount of methanol and 2-propanol in the gradient, the baseline drifted. This shift in baseline is perhaps due to increased viscosity of the mobile phase as the amount of solvent B is increased. This figure also showed the broadening of the base of zearalenone, zearalanone, zeranol and zearalenol peaks and indicated stronger interaction of these compounds with the support than oestradiol, DES and oestrone. A higher concentration of more polar B mobile phase was necessary to elute the former compounds, whereas oestradiol, DES and oestrone were also eluted in less than 20 min by a gradient of hexane and hexane:methanol:2-propanol (85:15:5, v/v) which is less polar than mobile phase B used in this study. In figure 3, the elution time of a compound varied when chromatographed individually compared to a faster elution time when analysed as a component of a mixture. This suggests that there is an interaction among the compounds which changes their behaviour with the surface of the solid support, resulting in alteration of their solubility with the mobile phase. Table 1 shows the mean elution times of the seven oestrogens as analysed individually or as a component of a mixture with coefficients of variation of 2–4% for individually chromatographed

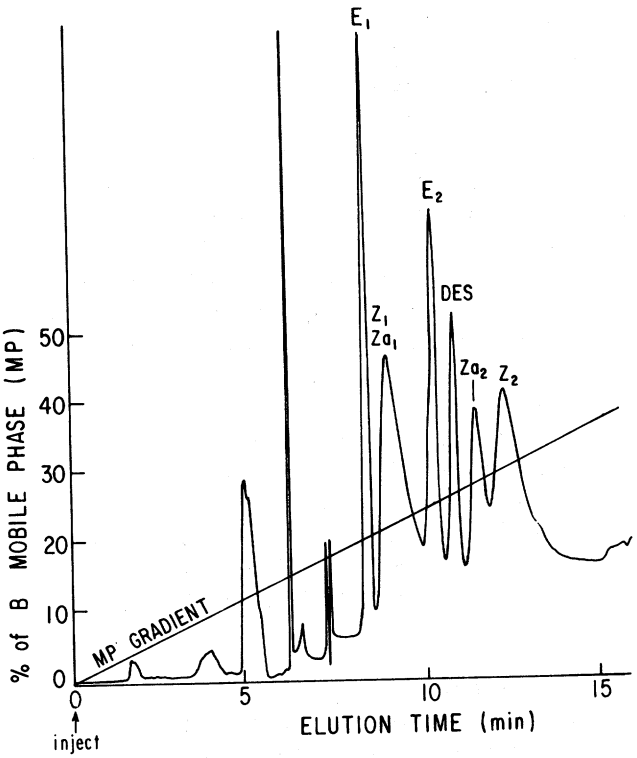

Figure 2. A seven-component HPLC analysis of anabolic oestrogens: oestrone (E_1), zearalenone (Z_1), zearalanone (Za_1), oestradiol (E_2), diethylstilboestrol (DES), zeranol (Za_2) and zearalenol (Z_2).

compounds and <1% for compounds chromatographed as a mixture. The greater variability of the individually analysed compounds can be attributed to a wider period when the analysis was made vs. analysis of the mixture in one week. *trans*-DES (*t*-DES) when chromatographed individually showed a second peak which is attributed to its isomerized form *cis*-DES (*c*-DES). This second peak develops as *t*-DES is stored in

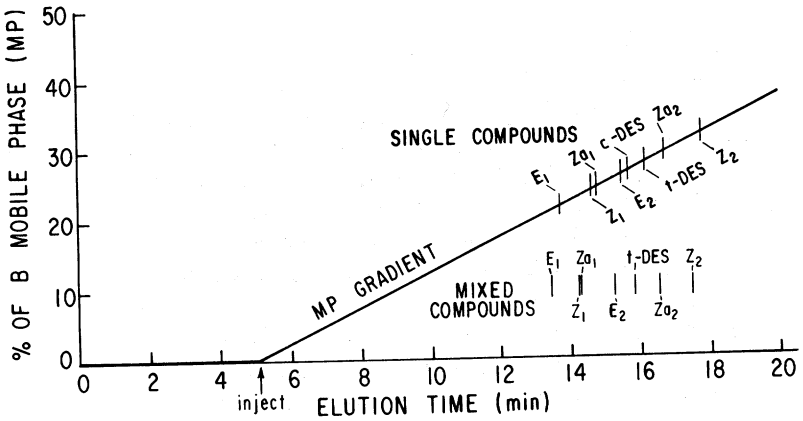

Figure 3. HPLC elution pattern of anabolic oestrogens analysed as single or mixed components of oestrone (E_1), zearalenone (Z_1), zearalanone (Za_1), *cis*-diethylstilboestrol (*c*-DES), *trans*-diethylstilboestrol (*t*-DES), oestradiol (E_2) and zeranol (Za_2).

Table 1. Reproducibility of elution times (min) of the anabolic oestrogens analysed by HPLC-UV.

	Oestrone	Zearalenone	Zearalanone	Oestradiol	*c*-Des	*t*-Des	Zeranol	Zearalenol
Individually chromatographed								
N	5	6	2	9	4	8	6	1
Mean elution time (min)[a]	8·72	9·61	9·74	10·47	10·60	11·13	11·70	12·76
SD	0·27	0·31	0·20	0·21	0·42	0·37	0·29	—
% CV	3·0	3·2	2·0	2·0	4·0	3·3	2·5	—
Chromatographed as a mixture								
N	3	3	2	3	—	3	2	3
Mean elution time (min)[a]	8·43	9·24	9·26	10·25	—	10·81	11·54	12·48
SD	0·02	0·07	0·08	0·06	—	0·06	0·03	0·12
% CV	0·02	0·76	0·86	0·59	—	0·55	0·26	1·0

[a] Corrected for 5 min stabilization prior to sample injection.
CV = coefficient of variation.

solvent at 4°C and increases with time. Therefore, *t*-DES standard must be freshly prepared or kept at −20°C for storage. When a binary mixture of DES and 17*β*-oestradiol were chromatographed, *c*-DES co-eluted with oestradiol. *c*-DES was detectable when higher amounts (25 or 50 ng) of *t*-DES were analysed.

Linearity of the parent compounds of oestrogens (oestradiol, zearalezone, *t*-DES or zeranol) were determined by analysing 5, 10, 25 or 50 ng. Oestradiol and zeranol were detectable at 5 ng, and *t*-DES and zearalenone were detectable at 10 ng. These detectabilities were based on peak heights at least twice greater than background. The regression lines were $y=0{\cdot}997+0{\cdot}0234x$, $r=0{\cdot}996$ for oestradiol; $y=0{\cdot}0313+0{\cdot}0209x$, $r=0{\cdot}997$ for zearalenone; $y=0{\cdot}0580+0{\cdot}0184x$, $r=0{\cdot}990$ for *t*-DES; and $y=0{\cdot}0132+0{\cdot}0137x$, $r=0{\cdot}999$ for zeranol. Recovery of the standards analysed by HPLC was approximately 80%.

Avian tissue extracts spiked with oestradiol and zeranol at 50, 100 or 200 ppb and cleaned up with the dual columns of alumina and ion-exchange prior to HPLC analysis showed that the oestrogens were detectable when an equivalent of 10 ng were injected

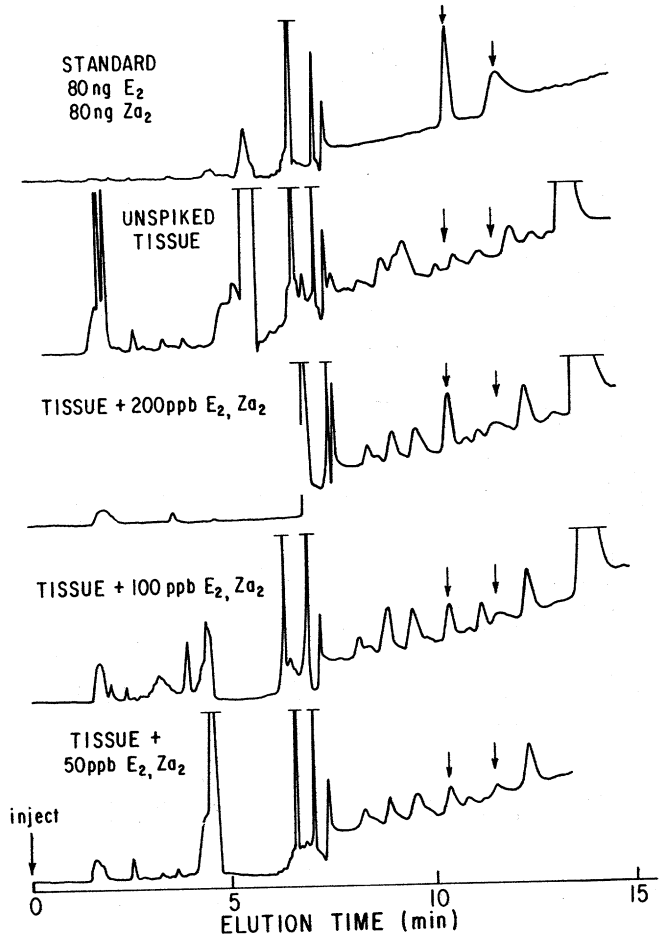

Figure 4. HPLC chromatograms of avian muscle tissue spiked with 50, 100 or 200 ppb oestradiol and zeranol and cleaned up through dual columns of basic alumina and phosphate-exchanged AGMP-1 resin. A theoretical equivalent of 10, 20 or 40 ng/20 μl oestradiol and zeranol in tissue samples were injected.

into the column (figure 4). The peak height or area of oestradiol in this figure also suggests that oestradiol can be detected below 10 ng. Recoveries were $\geqslant$86% for oestradiol and $\geqslant$83% for zeranol. There were no interfering peaks where oestradiol or zeranol eluted. Extracts of avian tissues spiked with 125 ng DES or zearalenone at 50 ppb also showed no interfering peaks with DES or zearalenone using the alumina and ion-exchange clean-up procedure. Injection of an equivalent of 10 ng/20 μl demonstrated the minimum detectable amounts for DES (figure 5*a*) or zearalenone (figure 5*b*) with recoveries of 60% and 100%, respectively.

Oestradiol can be used as internal standard for the analysis of other oestrogens, since oestradiol is stable and also possesses a highly reproducible elution pattern and a relatively high absorbance. A binary mixture of oestradiol and other anabolic oestrogen may be useful as standards in monitoring anabolic drugs as oestradiol is endogenous in animals and may be detected along with other anabolics. Analysis of tissue extracts spiked with a mixture of anabolics was not carried out in this study because in reality a farmer may treat an animal with only one anabolic oestrogen and not a combination of these compounds.

The described method demonstrated that anabolic oestrogens and structurally related mycotoxins can be separated by HPLC and detected by ultraviolet detection at low nanogram (*c.* 10 ng) levels when samples are spiked with low ppb (*c.* 50) of

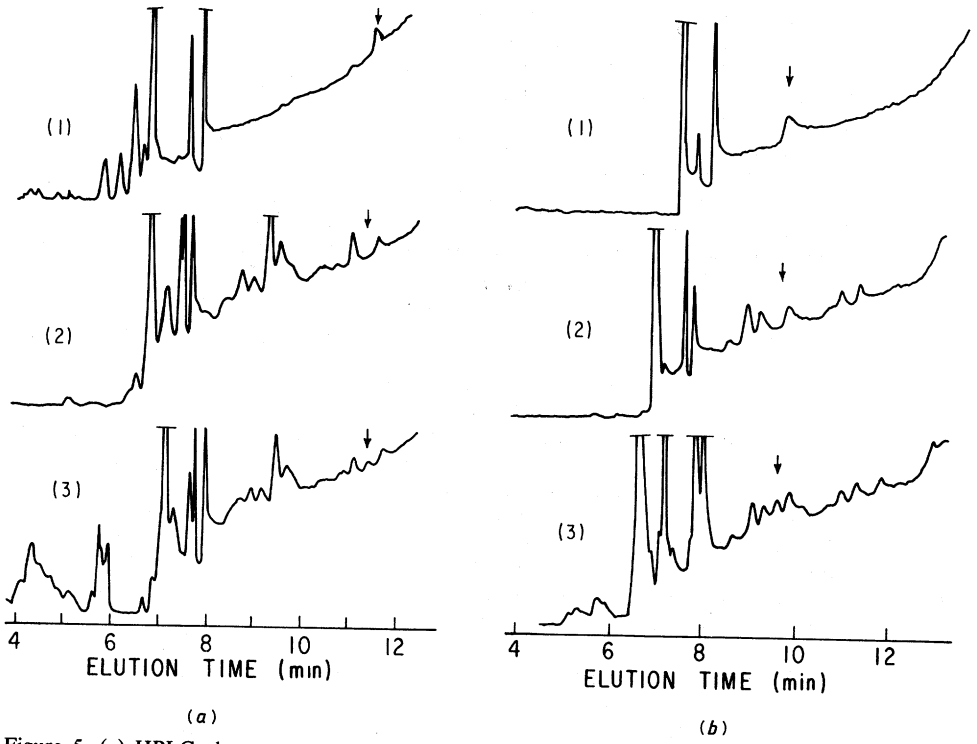

Figure 5. (*a*) HPLC chromatograms of (1) 10 ng DES standard, (2) unspiked chicken muscle extract purified in alumina and ion-exchange microcolumns, and (3) column-purified chicken extract spiked with 50 ppb DES injecting an equivalent of 10 ng/20 μl. (*b*) HPLC chromatograms of (1) 10 ng zearalenone standard, (2) unspiked chicken muscle extract purified in alumina and ion-exchange microcolumns, and (3) column-purified chicken extract spiked with 50 ppb zearalenone injecting an equivalent of 10 ng/20 μl.

oestrogens. Extraction, purification and HPLC analysis of six duplicate samples can be achieved in $1\frac{1}{2}$ days and should be applicable to screen oestrogens in cattle and sheep tissues. This method can be used to complement immunoassay methods or it can be employed as a preparatory method to fractionate samples so the identity of suspected compounds can be confirmed by mass spectrometry.

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References

BALDWIN, R. S., WILLIAMS, R. D., and TERRY, M. K., 1983, Zeranol: a review of the metabolism, toxicology and analytical methods for detection of tissue residues. *Regulatory Toxicology and Pharmacology*, **3**, 9–25.

BETINA, V., 1984, Zearalenone and brefeldin A. In: *Mycotoxins: Production, Isolation, Separation and Purification*, edited by V. Betina (New York: Elsevier) pp. 237–258.

Code of Federal Regulation, 1984, Part 21, Sections 522, 556.

FRISCHKORN, C. G. B., FRISCHKORN, H. E., and OHST, I. M., 1978, Simultaneous determination of the anabolic agent zeranol and its metabolite zearalanone by high performance liquid chromatography (HPLC). *Lebbensmittel Untersuchung und Forschung*, **167**, 7–10.

GROHMAN, H. G., JORDAN, S., and STAN, H. J., 1982, High pressure liquid chromatography of steroids. *Fresenius Zeitschrift für Analytische Chemie*, **311**, 399–400.

HAGLER, W. M., DANKO, G., HORVATH, L., PALYSIUK, M., and MIROCHA, C. J., 1980, Transmission of zearalenone and its metabolite into ruminant milk. *Acta Veterinaria Academiae Scientiarum Hungaricae*, **28**, 209–216.

HEFTMAN, E., and HUNTER, I. R., 1979, High pressure liquid chromatography of steroids. *Chromatographic Reviews*, **165**, 283–299.

HERRICK, J. B., 1984, *Cattle Disease Guide, Feedstuffs Reference Issue*, **6** (30), 112.

HOLDER, C. L., NONY, C. R., and BOWMAN, M. C., 1977, Trace analysis of zearalenone and/or zearalanol in animal chow by high pressure liquid chromatography and gas liquid chromatography. *Journal of the Association of Official Analytical Chemists*, **60**, 272–278.

HURD, R. N., 1977, Structure activity relationships in zearalenones. In: *Mycotoxins in Human and Animal Health*, edited by J. V. Rodricks, C. W. Heaseltine and M. A. Mehlman (Illinois: Pathotox Publishers) pp. 379–392.

IARC, 1979, *IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* (Lyon: International Agency for Research on Cancer).

JANSEN, E. H. J. M., BOTH-MIEDEMA, R., VAN BLITTERSWIJK, H., and STEPHANY, R. W., 1984, Separation and purification of several anabolics present in bovine urine by isocratic high performance liquid chromatography. *Journal of Chromatography*, **299**, 450–455.

LIN, J. T., 1984, Separation of steroid hormones by HPLC. *Liquid Chromatography*, **2**, 135–138.

LOESCH, C. H., and SIMON, W., 1983, Use of a stationary phase with a silica-bonded electrically neutral non-macrocyclic ligand for HPLC separation of steroids. *Chromatographia*, **17**, 669–672.

MEDINA, M. B., and SCHWARTZ, D. P., 1986, Isolation of estrogens in bovine plasma and tissue extracts using alumina and ion-exchange microcolumns. *Journal of Agricultural and Food Chemistry* In Press.

MIROCHA, C. J., SCHAUERHAMER, B., and PATHRE, S. V., 1974, Mycotoxins: isolation, detection and quantitation of zearalenone in maize and barley. *Journal of the Association of Official Analytical Chemists*, **57**, 1104–1110.

MIROCHA, C. J., PATHRE, S. V., and CHRISTENSEN, C. M., 1977, Zearalenone. In: *Mycotoxins in Human and Animal Health*, edited by J. V. Rodricks, C. W. Hesseltine and M. A. Mehlman (Illinois: Pathotox Publishers) pp. 345–364.

MIROCHA, C. J., SCHAUERHAMER, B., CHRISTIANSEN, C. M., NIKU-PAAVOLA, M. L., and NUMI, M., 1979, Incidence of zearalenol (*Fusarium* mycotoxin) in animal feed. *Applied and Environmental Microbiology*, **38**, 749–750.

PALYSIUK, M., HARRACH, B., MIROCHA, C. J., and PATHRE, S. V., 1980, Transmission of zearalenone and zearalenol into porcine milk. *Acta Veterinaria Academiae Scientarium Hungaricae*, **28**, 217–222.

ROOS, R. W., 1980, High pressure liquid chromatographic separation and identification of estrogens. *Journal of the Association of Official Analytical Chemists*, **63**, 80–87.

RYAN, J. J., 1976, Chromatographic analysis of hormone residues in food. *Journal of Chromatography*, **127**, 53–89.

SCOTT, P. M., PANALAKS, T., KANHERE, S., and MILES, W. F., 1978, Determination of zearalenone in cornflakes and other corn-based foods by thin layer chromatography, high pressure liquid chromatography and gas liquid chromatography/high resolution mass spectrometry. *Journal of the Association of Official Analytical Chemists*, **61**, 593–600.

TRENHOLM, H. L., WARNER, K. M., and FARNWORTH, E. R., 1981, High performance liquid chromatographic method using fluorescence detection for qualitative analysis of zearalenone and α-zearalenol in blood plasma. *Journal of the Association of Official Analytical Chemists*, **64**, 302–310.

TURNER, G. V., PHILLIPS, T. D., HEIDELBAUGH, N. D., and RUSSELL, L. H., 1983, High pressure liquid chromatographic determination of zearalenone in chicken tissues. *Journal of the Association of Official Analytical Chemists*, **66**, 102–104.